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USE OF SEQUENCE SPECIFIC NUCLEASES FOR SITE SPECIFIC MODIFICATION OF PLANT GENOME FOR CROP IMPROVEMENT

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ABSTRACT

Crop improvement is a complex process, which not only involves selection or breeding for a desired trait, but also includes different types of agronomic practices for crop production. In most cases of crop improvement processes, there is an inclusion, exclusion, or modification of one or few genes that underlie the trait targeted for improvement in crop plants. Advancement in genetic engineering techniques provides researchers a great advantage in controlling expression of plant gene/genes in a desired manner. From random mutations of plant genome using different mutagens like EMS (Ethyl Methane Sulfonate), X-rays, gamma-rays, etc. to site specific genome modification has been achieved, which has put greater acceleration in understanding gene expression and its regulation. Advancement of techniques involving components like Transcription Activator-Like Effector Nucleases (TALENs), Zinc Finger Nucleases (ZFNs), RNAi/ antisense technology and more recently CRISPR/ Cas allows us to study the genes and manipulate them more precisely. This review article highlights the application of molecular techniques like ZFNs, TALENs and CRISPR/ Cas in site-specific modification of different plant genomes, which has made gene expression, and regulation studies faster, easier and more reliable. The recent modifications in CRISPR/Cas9 strategy which offer greater potential for crop genetic improvement and breeding are also highlighted.

KEYWORDS: Designed Nucleases; Genome; DNA Repair; Genome Editing; Mutation & DNA Cleavage

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Original Article

INTRODUCTION

There are several methods which are designed to study the gene expression as well as to manipulate the expression of the targeted gene. Among those methods, the manipulation and regulation of target gene by using uniquely designed nucleases are gaining popularity nowadays because of their simple design, easy use and target specific activity.

The alteration of plant genome in a target specific fashion utilizes different approaches such as oligonucleotide-directed mutagenesis, gene targeting by homologous recombination, site specific gene integration using recombinase and site specific genome modification using nucleases (Cardi and Stewart 2016). The alteration in plant genome by mutation leads to several outcomes such as gene up-regulation, down-regulation, and knockout, knockdown and so on or there is no effect on expression of gene *per se*, if the alteration is in non-functional or non-coding genomic region.

Genome engineering is now focusing on the precise modification of the plant genome by utilizing activity of nucleases which causes double stranded breakage (DSBs) of DNA and stimulate mechanisms which repair the

DSBs (Voytas 2013). Homologous recombination (HR) and non-homologous end joining (NHEJ) are the primary mechanisms involved in correcting DSBs. The NHEJ causes mutation like random insertion or deletion (InDels) or substitution and if it occurs in the coding region of the gene it can cause frame shift mutation, resulting in a target gene knockout. The other mechanism of DNA DSBs repair is HR which involves integration of donor DNA having homologous overhangs at the target site (Cristea *et al.* 2013). The mechanism of repairing DNA DSBs in plants is well discussed in review papers by Puchta (2005) and Waterworth *et al.* (2011). In order to create DSBs on the target site, one has to develop a nuclease which recognizes the target site and cleaves the DNA very specifically. Due to a series of research over a period of time, mostly related to prokaryotes and their infection and defense mechanism, scientists have come across different types of gene targeting mechanism and nucleus involved in it. These key discoveries have helped to develop nucleases consisting of DNA binding domain which are either guided by protein or by RNA to produce a site specific breakage. The end result is a suite of nucleases which cause DSBs at specific site more efficiently such as ZFNs, TALENs and CRISPR/ Cas (Fig. 1).

Plant Genome Modification by Zinc Finger Nucleases (ZFNs)

Zinc finger nucleases (ZFNs) are the chimeric protein fusion of DNA cleavage domain of nucleases, with the DNA binding domain of zinc-finger-based DNA recognition modules (Kim *et al.* 1996). These ZFNs helps in creating site specific breaks in ds DNA, without pre-engineered target site (Bibikova *et al.* 2003 and Urnov *et al.* 2005). The zinc fingers bind to specific nucleotide triplets. When the target sequence specific array of zinc fingers is fused with endonuclease domain, more commonly a non-specific cleavage domain from *Fok*I, a type IIS restriction endonuclease, the breakage at a desired site can be created (Fig. 2a) (Kim *et al.* 1996). Although it was controversial initially, the zinc finger design proved to be efficient in recognizing specific DNA sequences (Beerli *et al.* 2000; Beerli and Barbas 2002 and Gonzalez *et al.* 2010). Once the gene targeting mediated by ZFNs was proved to be efficient, it was followed by a series of experiments where ZFNs were used successfully for genome modifications of different plants (Table 1).

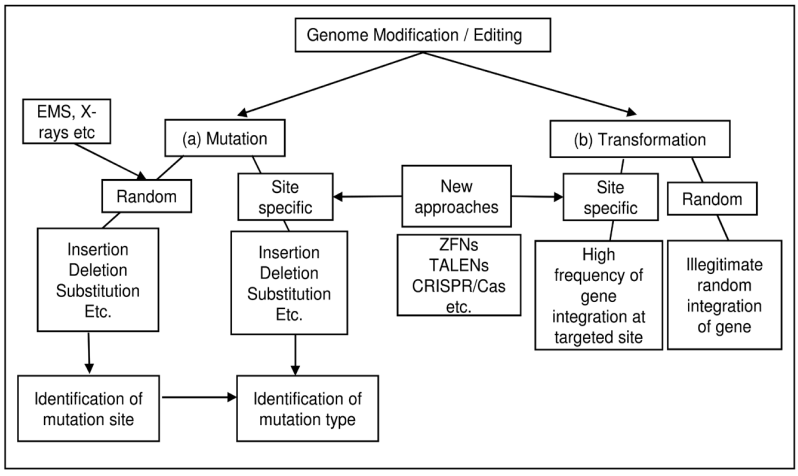

Figure 1: Random and Site Specific Genome Editing/ Modification Approaches

Transcription Activator-Like Effector Nucleases (TALENs) in Plant Genome Modification

TALEN is a chimeric protein fusion of DNA cleavage domain of nucleases with the DNA binding domain, similar to ZFNs. However, in TALENs the DNA binding domain is a Transcription Activator-Like Effector (TALE) DNA binding domain (Fig. 2b). TALEs are the proteins produced by bacteria, especially *Xanthomonas* sp. When causing

infection to plants (Boch and Bonas 2010). In the era of target genome editing for studying gene functions as well as manipulating specific genes for improving crop plants in a desired manner, the TALENs have shown great potential towards precise genome editing (Christian *et al.* 2010; Chaudhary *et al.* 2016). A TALE DNA binding domain is an array of highly conserved nearly identical tandem repeats of amino acid sequences and has simple repeat variable at two positions referred to as repeat variable di-residue (RVD). This RVD has a role in recognizing a specific DNA sequence (Boch *et al.* 2009; Moscou and Bogdanove 2009; Bogdanove *et al.* 2010; Bogdanove and Voytas 2011). By combining the sequence within the RVD, complementary to target sequence, the TALENs can be designed to target any region of the DNA for site specific cleavage. With all these discoveries, many organisms' genomes, including plant genomes have been modified using TALENs (Char *et al.* 2015). In plants, there are several reports where the scientists have successfully used engineered TALENs for regulating the expression of targeted gene/genes in a desired manner (Table 1).

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR/Cas) in the Plant Genome Modification

CRISPR has emerged as an efficient genome editing tool and tool for regulating targeted gene expression within a short period of discovery of its working principle. CRISPR is a genomic locus which was first discovered as part of adaptive immunity in bacteria and archaea (Deveau *et al.* 2010). CRISPR locus consists of tandem direct repeats sequences and spacer between the repeat sequences which are derived from the invading elements and are called as CRISPR RNA (crRNA) (Kim and Kim 2014). The crRNA combines with another small RNA molecule, known as transactivating CRISPR RNA (tracrRNA), which is complementary to crRNA repeats and helps in a processing of crRNA. After processing, crRNA and tracrRNA complex activates and guides the CRISPR associated (Cas) nucleases (generally Cas9), to the target site (Fig. 2c). The targeting of Cas nuclease into specific site by crRNA and tracrRNA, leads to cleavage of homologous double stranded DNA sequences, termed as protospacers of the invading elements (Barrangou *et al.* 2007). Downstream of the protospacers sequences, the conserved sequence usually 5'-NGG-3' (N= A or T or G or C) or less frequently 5'-NAG-3' termed as protospacer adjacent motif (PAM) is present, which is essential for Cas mediated cleavage of the target site (Gasiunas *et al.* 2012; Jinek *et al.* 2012; Hsu *et al.* 2013). For applications in crop improvement, the crRNA can be engineered to target any DNA sequences, by taking the spacer sequences from the target region preceding the PAM sequence. For the ease of transformation and to increase its efficiency, crRNA and tracrRNA can be fused artificially to form single guide RNA (Mali *et al.* 2013; Qi *et al.* 2013). The CRISPR/ Cas9 (CRISPR-associated) is the most widely used RNA-guided editing system. It is a type II system from *Streptococcus pyogenes* and within a short period of discovery of its efficient genome editing ability, CRISPR/ Cas9 has become a common tool for genome editing and tool for regulating targeted gene expression in different plants (Table 1) and as well as in other organisms.

The CRISPR/Cas9 based genome editing in plants as a tool for crop improvement has several drawbacks. This strategy in plants usually depends on stable transformation with constructs expressing Cas9 and sgRNA(s), mostly using *Agrobacterium*-mediated transformation which is time-consuming. Recently, virus based vector systems have been proposed as an alternative strategy for delivery of expression cassettes for genome editing in plants (Ali *et al.* 2015; Honig *et al.* 2015). Identification of a suitable target region and design of sgRNA is one of the key steps for CRISPR/Cas9 strategy as it is known that different guide sequences in gRNA have variable efficiency in genome editing as well as lead to off-target mutations (Bae *et al.* 2014). To address this, several Bioinformatics tools are available (https://omictools.com/crispr-cas9-category). Recently, a new tool, CRISPR-P version 2.0 (CRISPR-P 2.0) has become freely available (http://cbi.hzau.edu.cn/CRISPR2/). It provides web services for computer-aided design of sgRNA with

minimal off-target potentials (Liu *et al.* 2017). This tool accepts 49 different plant genomes as input and allows design of sgRNA using gene locus tag, genomic position, or genomic sequence as input. The other drawback with CRISPR/Cas9 is the use of the process of NHEJ, which generates knock-out and loss-of-function mutants. Many of the agriculturally important traits are governed by single-nucleotide polymorphism or by dominant gain-of-function point mutations (REF). The mutants generated by CRISPR/Cas9 though valuable in understanding gene functions have somewhat limited application in crop improvement programmes. Cas9 has now been engineered as a DNA-binding protein, without the nuclease activities (dCas9), which has D10A and H840A mutations. A gRNA molecule brings dCas9 protein, to a specific DNA target, thus offering unprecedented opportunities to modify structures or properties of DNA/chromatin, near the target site (Komor *et al.* 2016a). Recently, dCas9 fused with a cytidine deaminase enzyme was shown to have the ability to convert C to T (or G to A), within a window of approximately five nucleotides, providing a tool for "base editing" thereby, increasing the scope and efficiency of point mutations (Komor *et al.* 2016b). This edited Cas9 (dCas) system now has been successfully utilized, for manipulating starch content in rice (Li *et al.* 2017). Most of the initial CRISPR/Cas9 systems reported in plants could only modify one or few targets. The availability PCR-based procedure to rapidly generate multiple sgRNA expression cassettes and assembly into the binary CRISPR/Cas9 vectors in one round of cloning, now allows for convenient and high-efficiency multiplex genome editing in monocot and dicot plants (Ma *et al.* 2015b). Recently, precise point mutations to three target sites in rice have been successfully introduced, thus providing a feasible and effective tool for targeted base editing in a crop plant (Li *et al.* 2017).

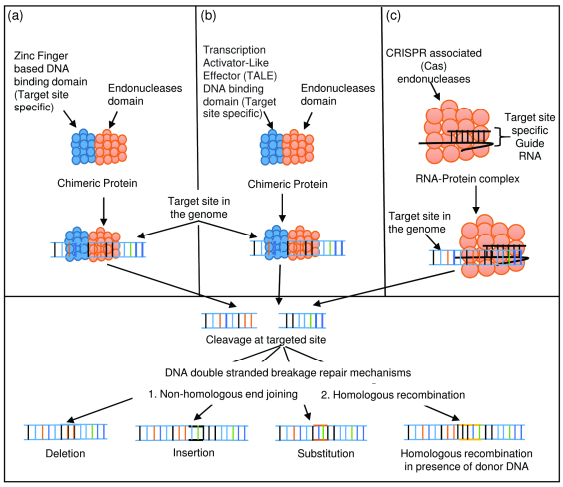

Figure 2: Mode of Action of (a) Zinc Finger Nucleases (ZFNs) (b) Transcription Activator-Like Effector Nucleases (TALENs) and (c) Clustered Regularly Interspaced Short Palindromic Repeats/Cas (CRISPR/Cas)

Table 1: List of some of the Targeted Genome Modifications in Different Plants using Nucleases

Plant Species	Types of Modification	Target Site or Gene/Genes	Based on	References
Arabidopsis thaliana	Site specific mutation	*Eco*RI restriction site	ZFNs	Lloyd *et al.* 2005
Nicotiana tabacum	Gene integration	*β-glucuronidase* (*GUS*) and *neomycin phosphotransferase* (*NPTII*) fusion	ZFNs	Wright *et al.* 2005
Nicotiana tabacum	Gene correction	*Sulfonylurea Receptor* (*SuRA and SuRB*)	ZFNs	Townsend *et al.* 2009

Table 1: Contd.,				
Zea mays	Gene replacement	*Inositol 1,3,4,5,6-pentakisphosphate 2- kinase* (*IPK1*) replaced by *Phosphinothricin Acetyl Transferase* (*PAT*) gene	ZFNs	Shukla *et al.* 2009
Arabidopsis thaliana	Gene replacement	*Hygromycin phosphotransferase* (*HPT*) replacing the *Green Fluorescent Protein* (*GFP*) gene	ZFNs	Pater *et al.* 2009
Arabidopsis thaliana	Gene correction	*β-Glucuronidase* (*GUS*)	ZFNs	Tovkach *et al.* 2009
Nicotiana tabacum	Deletion of intervening sequence	*Green Fluorescent Protein* (*GFP*) gene partial fragments	ZFNs	Cai *et al.* 2009
Arabidopsis thaliana	Gene integration	*Phosphinothricin Acetyl Transferase* (*PAT*) gene	ZFNs	Cai *et al.* 2009
Arabidopsis thaliana	Deletion and substitution	*ABA-Insensitive4* (*ABI4*)	ZFNs	Osakabe *et al.* 2010
Nicotiana tabacum	Transgene deletion	*β-Glucuronidase* (*GUS*)	ZFNs	Petolino *et al.* 2010
Arabidopsis thaliana	Gene disruption	*Alcohol dehydrogenase-1* (*ADH1*) and *Transparent testa-4* (*TT4*) genes	ZFNs	Zhang *et al.* 2010
Glycine max	Gene disruption	*DICER-Like* (*DCL*), *RNA dependent RNA polymerase (RDR)* and *HUA enhancer1* (*HEN1*)	ZFNs	Curtin *et al.* 2011
Arabidopsis thaliana	Gene integration	*Protoporphyrinogen oxidase* (*PPO*)	ZFNs	Pater *et al.* 2013
Solanum lycopersicum	Gene disruption	*Procera* (*PRO*)	TALENs	Lor *et al.* 2014
Glycine max	Gene knockout	*Fatty acid desaturase 2-1A (FAD 2-1A)* and *FAD2-1B*	TALENs	Haun *et al.* 2014
Oryza sativa	Gene knockout	*Lipoxygenase 3* (*LOX3*)	TALENs	Ma *et al.* 2015
Triticum aestivum	Insertion mutation	*Mildew resistance locus* (*MLO*)	TALENs	Wang *et al.* 2015
Solanum tuberosum	Base deletion	*Acetolactate synthase* (*ALS*)	TALENs	Nicolia *et al.* 2015
Oryza sativa	Gene knockout	*Oryza sativa betaine aldehyde dehydrogenase 2* (*BADH2*)	TALENs	Shan *et al.* 2015
Zea mays	Gene disruption	*Glossy2* (*gl2*) locus	TALENs	Char *et al.* 2015
Oryza sativa	InDel	*Oryza sativa carbon starved anther* (*OsCSA*), *Oryza sativa photo-sensitive male sterile 3* (Os*PMS3*), *Oryza sativa drought and ethylene responsive factor 1* (*OsDERF1*), *Oryza sativa grain number 1a (OsGN1a)*, *Oryza sativa tRNA-specific adenosine deaminase1* (*OsTAD1*), *OsMST7* and *OsMST8*,	TALENs	Zhang *et al.* 2016
Oryza sativa	Gene replacement Homologous recombination	*OsALS* gene replaced by mutated *OsALS** gene	TALENs	Li *et al.* 2016
Solanum tuberosum	Gene knockout	*Vacuolar invertase* (*VInv*)	TALENs	Clasen *et al.* 2016
Nicotiana benthamiana	Insertion	*Acetolactate synthase* (*ALS*)	TALENs	Stoddard *et al.* 2016
Saccharum spp. hybrids	InDel	*Caffeic acid O-methyltransferase* (*COMT*)	TALENs	Jung and Altpeter 2016
Solanum tuberosum	Homologous recombination	Site adjacent to *Ubi7* (*ubiquitin*) promoter	TALENs	Forsyth et al. 2016

Table 1: Contd.,				
Oryza sativa	Gene disruption	*Oryza sativa myeloblastosis 1*(OsMYB1), *OsMYB5*, *Oryza sativa young seedling albino* (*OsYSA*), *Oryza sativa rice outmost cell-specific gene 5* (OsROC5), *OsDERF1*, *Oryza sativa phytoene desturase* (*OsPDS*), *Oryza sativa 5-enolpyruvylshikimate-3-phosphate synthase* (*OsEPSPS*), *Oryza sativa photo-sensitive male sterile* (*OsPMS*), *Oryza sativa signal peptide peptidase* (*OsSPP*), *Oryza sativa MutS homolog1* (*OsMSH1*)	CRISPR/Cas9	Zhang *et al.* 2014
Solanum lycopersicum	Gene disruption	*Solanun lycopersicum Argonaute 7*(*SlAGO7*)	CRISPR/Cas9	Brooks *et al*. 2014
Arabidopsis thaliana	InDel	*Flowering locus T* (*FT*), *Squamosa promoter binding protein like 4* (*SPBP LIKE*4)	CRISPR/Cas9	Hyun *et al.* 2015
Oryza sativa	InDel	*Oryza sativa flowering locus T-like* (*OsFTL*), *Oryza sativa Glutathione S-transferase U* (*OsGSTU*), *Oryza sativa multidrug resistance-associated protein 15* (*OsMRP15*) and *OsWAXY*	CRISPR/Cas9	Ma *et al.* 2015
Oryza sativa	InDel	*Oryza sativa alternative oxidase 1a (OsAOX1a)*, *OsAOX1b*, *OsAOX1c* and *Oryza sativa bentazon-sensitive-lethal* (*OsBEL*)	CRISPR/Cas9	Xu *et al.* 2015
Arabidopsis thaliana	Gene disruption	*Eukaryotic translation initiation factor* paralogue *4E* (*eIF(iso)4E*)	CRISPR/Cas9	Pyott *et al.* 2016
Oryza sativa	Gene disruption	*Oryza sativa starch branching enzyme llb* (*OsSBEIIb*)	CRISPR/Cas9	Baysal *et al*. 2016
Oryza sativa	Gene disruption	*Grain number 1a* (*GN1A*), *Dense and erect panicle 1* (*DEP1*), *Grain size 3* (*GS3*), and *Ideal plant architecture 1* (*IPA1*)	CRISPR/Cas9	Li *et al.* 2016
Cucumis sativus	Gene disruption	*Eukaryotic translation initiation factor 4E* (*eIF4E*)	CRISPR/Cas9	Chandrasekaran *et al*. 2016
Oryza sativa	Point mutation	*Oryza sativa phytoene desturase* (*OsPDS*) and *Oryza sativa starch branching enzyme llb* (*OsSBEllb*)	CRISPR/Cas9	Li *et al*. 2017
Zea mays	Gene disruption	*Zea mays argonaute 18a* (*ZmAGO 18a*), *ZmAGO 18b*, *ZmAGO a1 and ZmAGO a4*	CRISPR/Cas9	Char *et al*. 2017

Advantages of Designed Nucleases in Genome Manipulation

The development of designed nucleases has made it possible, to manipulate the genome of organisms in a target specific manner. Although the designing of guide protein in ZFNS and TALENs is sophisticated and tedious, the manipulation of genome with these techniques is precise. But the advancement of CRISPR/Cas has overshadowed the application of ZFNs and TALENs in genome modification, because of its simple design and efficiency. The CRISPR/Cas

techniques have gained rapid popularity among the researcher's associated with genome manipulation within a short period of its discovery and has been utilized for different purposes from plant genome modification to human genome editing.

The ability of designed nucleases to create DSBs at targeted site can be used not only in creating mutation in the plant genome, but also can be used in providing plant resistance against biotic stress like virus infection (Romay and Bragard 2017). The site specific integration of transgenes, has been achieved through homologous recombination with the application of designed nucleases. However, the integration of the transgene, through illegitimate recombination is not yet under control. Therefore, more research is required to find out the mechanism to control the random integration of transgene when designed nucleases are used for site specific integration of the transgenes. The transformation of sequence specific nucleases (SSNs) coding transgenes into plant cells/tissues is generally DNA based, which is an effective means of transferring SSNs into plant cells. However, the DNA gets randomly integrated into the plant genome that can cause unintentional silencing of the gene among other kinds of detrimental effects. Stoddard *et al*. (2016) reported RNA based delivery system where the mRNA instead of DNA coding for SSNs is directly transformed into the plant cells. The translation of that particular mRNA leads to production of SSNs which then creates DSBs at targeted site. This technique will eliminate the problem of random integration of transgene as the mRNA won't be able to integrate itself in the genome. It also reduces the chances of off target mutation due to the continuous expression of SSNs from DNA based constructs. Both DNA and RNA based delivery system of SSNs can be used in variety of crop improvement application from crop bio-fortification to inactivation of an unwanted gene such as the selectable marker genes used in transgenic which is one of the reasons for the unacceptability of transgenic crops. These genes can be inactivated or removed by using designed nucleases by utilizing the guide RNA (gRNA) sequences targeting the selectable marker genes and thereby, producing selectable marker free transgenic crop plants.

Advantages of the CRISPR/Cas9 System Compared to Other Nucleases

The CRISPR/Cas9 system does not require any protein engineering steps. Furthermore, only 20 nucleotides in the gRNA sequence are needed to impart target specificity and this allows assembly of large gRNA libraries for high-throughput functional genomics application. Unlike ZFNs and TALENs, CRISPR/Cas9 system can cleave methylated DNA in human cells (Hsu *et al.* 2013) and in plants like *Arabidopsis* (Lowder *et al.* 2015), allowing genomic modifications that are beyond the reach of other nucleases (Bortesi and Fischer 2015). The ease of multiplexing with the ability to simultaneously introduce DSBs or point mutations at multiple points to edit several genes at the same time is the main practical advantage of CRISPR/Cas9. This approach of genome editing allows precise and predictable modifications directly in elite cultivars or accessions, saving the time-consuming backcrossing procedure. The CRISPR/Cas9 system should provide a more efficient approach to pyramid different genes as it is possible to modify multiple traits simultaneously (Bortesi and Fischer 2015). The success of this technology is also reported from complex genomes like maize (*Zea mays*), tomato (*Solanum lycoperscicum*) among others (Table 1; for details see Bortesi and Fischer 2015). Also the open access to all the tools like plasmids and web resources, for selecting gRNA sequences and predicting sequence specificity in the case of the CRISPER, has led to widespread use of this technology.

CONCLUSIONS

The simplicity, accessibility and affordability of screening of loss-of-function mutants on a genomic scale in any plant are the key advantages of CRISPR/Cas9 technology. This technology will enhance and facilitate both forward and

reverse genetic in any species including model species. With the recent advances in modification in the strategy, it will now be possible to minimize off-target mutations and introduce point mutations, thereby, increasing the scope of application of CRISPR/Cas9 for crop improvement. Given the number of publications (over 325 articles on applications of CRISPR/Cas9 alone in last 2 yrs; www. cell.com/nucleus-CRSPR) on of CRISPR/Cas9 technology since the first reports of genome editing only 4 years ago, further advances in our understanding and application of this system are to come rapidly. The feasibility of having transgene-free plants with the desired phenotype through CRISPR/Cas9-mediated method (Woo *et al.* 2015), opens a new door for crop improvement by editing genomes and still circumvents traditional regulations, on genetically modified organisms.

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